Agonists/Antagonists of the Insect Kinin and Pyrokinin/PBAN Neuropeptide Classes as Tools for Rational Pest Control

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1 Introduction

While insect neuropeptides of the insect kinin (IK) and pyrokinin/PBAN (PK/PBAN) family are both potent and specific, these molecular messengers are not suitably designed to be effective either as pest insect control agents and/or tools for insect neuroendocrinologists. Neuropeptides are rapidly degraded by peptidases in the hemolymph and tissues within insects and generally exhibit poor bioavailability (Nachman et al. 2001, 2002a, b). The development of potent agonists and antagonists with enhanced biostability and bioavailability can overcome these limitations and can represent a key step in the development of pest management techniques based on neuropeptide analogs capable of disrupting critical life processes regulated by the IK and PK/PBAN families. In two separate sub-sections, a review is presented on what is known about chemical, conformational, and stereochemical aspects of the interaction of the IK and PK/PBAN families with their putative receptors, and how this knowledge can be harnessed to design and develop biostable mimetic analogs that retain an ability to bind, and potentially activate, those receptors. Strategies for the modification of the PK/PBAN neuropeptides to enhance bioavailability characteristics are also discussed and should be applicable to other insect neuropeptide classes.

2 Insect Kinin Neuropeptide Family

Insect neuropeptides of the insect kinin (IK) class share a common C-terminal pentapeptide sequence Phe_1-Xaa_2-Xaa_{23}-Trp_4-Gly_5-NH_2 (Xaa_2 = His, Asn, Phe, Ser or Tyr; Xaa_3 = Pro, Ser or Ala). They have been isolated from a number of insects, including species of Dictyoptera, Lepidoptera, Diptera and Orthoptera. The first members of this insect neuropeptide family were isolated on the basis of their

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I. Ishaaya and A.R. Horowitz (eds.), *Biorational Control of Arthropod Pests*,
DOI 10.1007/978-90-481-2316-2_2,

ability to stimulate contractions of the isolated cockroach hindgut (Holman et al. 1987, 2002a; Coast 1998), but they are also potent diuretic peptides that stimulate the secretion of primary urine by Malpighian tubules, organs involved in the regulation of salt and water balance (Nachman et al. 1990, 2002a; Coast et al. 1990, 1998). In addition, the IK have been implicated in the regulation of digestive enzyme release (Sajjaya et al. 2001; 2002 Harshini et al., 2003). IK, and/or analogs, have also been reported to inhibit weight gain by larvae of the tobacco budworm (*Heliothis virescens*) and corn earworm (*Helicoverpa zea*) (Seinsche et al., 2000; Nachman et al., 2002a,2003), both important agricultural pests.

2.1 *Chemical, Conformational and Stereochemical Aspects of IK/Receptor Interaction*

Myotropic and diuretic assays of tissues in vitro, as well as assays using stably expressed IK receptors, show that the full biological activity of the IK resides in the C-terminal pentapeptide, which is the active core (Nachman et al. 1991, 1993, 2003), with the exceptions of the housefly Malpighian tubule fluid secretion assay (Coast 2001) and an expressed IK receptor from the mosquito *Aedes aegypti*, where the C-terminal pentapeptide core is less potent than native peptides by several orders of magnitude. Diuretic, myotropic, and/or receptor-interaction activity in these assays is completely lost when the C-terminal amide of the insect kinins is replaced with a negatively charged acid moiety (Nachman et al. 1995). Within the core pentapeptide, the aromatic residues Phe_1 and Trp_4 are the most important for activity whereas a wide range of variability is generally tolerated at position 2, from acidic to basic residues and from hydrophilic to hydrophobic (Nachman et al. 1991, 1993b). The expressed IK receptor from the mosquito *A. aegypti* represents a particular exception, as it clearly prefers an aromatic residue in position 2, which is consistent with the presence of aromatic residues in position 2 of all three of the native aedeskinins (Taneja-Bageshwar et al. 2006).

NMR spectroscopic data and molecular dynamics calculations on an active head-to-tail, cyclic analog (*cyclo*[AFFPWG]) reveal the presence of two major turn types within the active core region of the IK (Roberts et al. 1997). The more rigid of the two conformations featured a *cis*Pro in the third position of a type-VI β-turn over core residues 1–4, or Phe-Phe-**Pro**-Trp. ROESY spectra supported a well-defined Cβ-*exo*/Cγ-*endo* pucker for the *cis*Pro ring that was observed in unrestrained molecular dynamics for this cyclic analog. The other less rigid turn system involved a *trans*Pro and encompassed residues 2–5, or Phe-**Pro**-Trp-Gly (Roberts et al. 1997).

In an effort to provide definitive evidence that the most populous *cis*Pro type VI β-turn over residues Phe1 through Trp4 represented the 'active conformation' for receptor interaction, IK analogs incorporating restricted conformation components that preferentially mimic a *cis* peptide bond and a type VI β-turn were synthesized and evaluated. NMR studies with IK analogs incorporating either the tetrazole or 4-aminopyroglutamate (APy), moieties that mimic one turn over the other, indicate

a predominant population of a β-turn involving the Phe_1 to Trp_4 region (Nachman et al. 2002b, 2004). These restricted conformation analogs in the natural L,L (or S,S) configuration demonstrate significant retention of both diuretic activity in the cricket Malpighian tubule fluid secretion assay and interact with the expressed IK receptor from the tick *B. microplus* (Taneja-Bageshwar et al. 2008a).

In a more detailed investigation, all four stereochemical variants of tetrazole and APy moieties were incorporated into the C-terminal region of the insect kinin sequence and evaluated in a cricket Malpighian tubule fluid secretion assay and an expressed IK receptor from the tick *Boophilus microplus*. The optimal stereochemistry for the two turn mimic moieties in both the cricket Malpighian tubule fluid secretion assay and expressed tick receptor for agonist activity was identified as (D,L) and (2R,4S)(Taneja-Bageshwar et al. 2008a), respectively; with APy analog Ac-RF[APy]WGa (*R,S*) demonstrating an EC_{50} of 7 nM in the cricket diuretic assay. In contrast, the (L,D)-tetrazole analog demonstrated an ability to antagonize the diuretic response of natural achetakinins (Kaczmarek et al. 2007) in the in vitro cricket diuretic assay, but was inactive on the expressed tick receptor. It was suggested that the change in stereochemistry of the α-carbon at the N-terminal end of the tetrazole moiety from L to D appears to inhibit the activation response by interfering with the electrostatic interaction that occurs between the side chains of the Phe_1 and Trp_4 that allows these two critical side chains to present an aromatic surface to the receptor (Nachman et al. 2004).

The critical nature of the sidechains of Phe_1 and Trp_4 and the *cis*Pro, type VI turn conformation to the activity of the IK was also confirmed by evaluation of a small series of pseudotetrapeptide analogs that featured only these minimal constructs. These 'minimalist' analogs, based on an amino piperidinone carboxylate scaffold, retained very weak, but statistically significant diuretic activity in the in vitro cricket Malpighian tubule secretion bioassay (Kamoune et al. 2005).

In summary, structure/conformation-activity data are consistent with a receptor interaction model for the IK in which the C-terminal pentapeptide region adopts a type VI turn over residues Phe_1 to Trp_4, and that the aromatic side chains of Phe_1 and Trp_4 are oriented towards the same region and interact with the receptor. Conversely, the side chain of residue 2 lies on the opposite face pointing away from the receptor surface, explaining why this position shows greater tolerance to modifications (Nachman et al. 2002b).

2.2 *Biostable, IK Analogs That Interact with Receptors and Bioassays*

Members of the IK family are inactivated by tissue-bound peptidases of insects. Two susceptible hydrolysis sites in insect kinins (Nachman et al. 2002a) have been reported. The primary site is between the Pro_3 and the Trp_4 residues, with a secondary site N-terminal to the Phe_1 residue in natural extended IK sequences. Experiments demonstrate that angiotensin converting enzyme (ACE) from the housefly can

cleave at the primary hydrolysis site, whereas neprilysin (NEP) can cleave insect kinins at both the primary and secondary hydrolysis sites (Nachman et al. 1990, 2002a ; Cornell et al. 1995; Lamango et al. 1996;).

Incorporation of β-amino acids can enhance both resistance to peptidase attack and biological activity (Cheng et al. 2001; Juaristi and Soloshonok 2005). Recent work has described the synthesis of a number of analogs of the IK C-terminal pentapeptide core in which the critical residues Phe_1, Pro_3 and Trp_4, and/or adjacent residues, are replaced with β_3-amino acid and/or their β_2-amino acid counterparts.

It was anticipated that incorporation of β-amino acids in key positions of the IK would afford some measure of resistance to hydrolysis by the peptidases that degrade and inactivate the natural peptides. Indeed, several analogs including Ac-R[β_3**Phe**]FF[β_3**Pro**]WGa (1577), and Ac-RF[β_3**Phe**]-[β_3**Pro**]WGa (1578) have been shown to exhibit significantly enhanced resistance to peptidases that attack at insect kinin susceptible sites (Zubrzak et al. 2007). Under conditions in which the native IK from *Leucophaea*, leucokinin-I, is degraded 100% by NEP and 82% by ACE, analog 1578 shows no degradation by NEP and only 4% by ACE. These analogs are also blocked at the N-terminus with an Ac group, which confers resistance to hydrolytic degradation by an additional class of peptidases, the aminopeptidases (Gregory et al. 1964).

A single-replacement β-amino acid analog Ac-RFF[β_3**Pro**]WGa (1460), that involves modification of the Pro_3, was the most potent of the β-amino acid analogs in both mosquito (*A. aegypti*) and tick (*B. microplus*) receptors (Taneja-Bageshwar et al. 2008b) (Table 1). Analog 1460 proved to be more active than the positive control agonist FFFSWGa in the mosquito, and considerably more active than the minimum active core sequence FFSWGa. In the tick, 1460 was equipotent with the positive control agonist FFFSWGa and more active than the C-terminal pentapeptide active core. Analog 1460 therefore is a non-selective, biostable agonist for these two receptor systems. However, it is clear that the mosquito receptor is considerably more sensitive to modifications at Trp_4 than the tick receptor. In particular, the mosquito receptor is intolerant to the replacement of Trp_4 with β_2Trp, as 1656 (Ac-RFFP[β_2**Trp**]Ga) is inactive (Taneja-Bageshwar et al. 2008b) (Table 1). This complete loss of activity by the β_2Trp analog 1656 in the mosquito receptor cell line may result from an increase in the distance between the α-carbons of the critical aromatic residues at positions 2 and 4 over what is found in either the natural peptide. On the contrary, the tick receptor remains relatively tolerant to the introduction of a methylene group between the α-carbon and the amino group of Trp_4, as the potency of 1656 was not statistically different from the C-terminal pentapeptide core analog FFSWGa, and the analog retained 57% of the maximal response (Taneja-Bageshwar et al. 2008b). By virtue of this difference in receptor-interaction requirements, analog 1656 is a selective agonist for the tick receptor.

The double replacement analog 1577 features modification of non-critical regions; the non-critical Pro_3 residue and the residue just outside of the critical C-terminal pentapeptide core region. Accordingly, this analog retains significant activity in both the mosquito and tick receptor assays, demonstrating potency that

exceeds that of the minimum active core and which is not significantly different from that of the positive control agonist FFFSWGa (Taneja-Bageshwar et al. 2008b). It is a nonselective, biostable agonist of both arthropod receptors. Double replacement analog 1578 features modification of non-critical residues Phe_2 and Pro_3 that effectively changes the distance between the sidechains of the critical residues Phe_1 and Trp_4. Like analog 1656, this modification is tolerated to a much greater extent by the tick receptor than the mosquito receptor. Consequently, the analog 1578 demonstrates retention of activity in the tick receptor assay, with a potency that is not significantly different from that of the active core analog FFSWGa; but remains essentially inactive in the mosquito receptor system (Taneja-Bageshwar et al. 2008b) (Table 1). Thus, biostable agonist analog 1578 demonstrates selectivity between the two expressed arthropod receptor cell lines.

The β-amino acid IK analogs have not as yet been evaluated in in vitro mosquito or tick diuretic assays. However, data for these analogs has been reported in an in vitro insect kinin Malpighian tubule fluid secretion assay from the cricket *Acheta domesticus* (Zubrzak et al. 2007). As with the two expressed arthropod insect kinin receptors, analog 1460 was the most potent β-amino acid analog in the cricket fluid secretion bioassay, demonstrating a potent EC_{50} of 0.03 nM and a 100% maximal response. This analog exceeds by an order of magnitude the activity of at least one of the native achetakinins, AK-III (EC_{50} = 0.3 nM), and essentially matches the activity of the most potent of achetakinins (Coast et al. 1990). As in both expressed arthropod receptor assays, the double-replacement analog 1577 retained potent activity in the cricket diuretic assay, with an EC_{50} value of 0.1 nM and gave a 100% maximal response (Zubrzak et al. 2007). Although inactive, or virtually so, in the mosquito IK receptor, analogs 1656 (EC_{50} = 4 nM; 97% maximal response) containing β_2Trp and double replacement 1578 (EC_{50} = 1 nM; 100% maximal response) retain activity in the in vitro cricket diuretic assay (Zubrzak et al. 2007). The assay results obtained with the β-amino acid IK analogs suggest that the receptor associated with the Malpighian tubules of the cricket is more similar to the expressed IK receptor from the tick than that of the mosquito.

Another set of IK analogs that demonstrate enhanced resistance to peptidases feature a replacement of the third position of the C-terminal pentapeptide core (Pro or Ser) with α-aminoisobutyric acid (Aib), a sterically hindered α,α-disubstituted amino acid which effectively protects the primary tissue-bound peptidase hydrolysis site (Nachman et al. 1997a, 2002a). Incorporation of a second Aib residue adjacent to the secondary peptidase hydrolysis site, as with analog **[Aib]**FS**[Aib]**WGa (781), further enhances biostability (Nachman et al. 2002a). In the cricket Malpighian tubule assay, analog 781 exceeds the potency of the native achetakinins by an order of magnitude. The di-substituted Aib-containing analog **[Aib]**FF**[Aib]**WGa (**K-Aib-1**) also demonstrates a potency that either matches or exceeds that of the positive control FFFSWGa and/or the native *Aedes*kinin-2 in recombinant tick and mosquito receptors, respectively (Fig. 1) (Taneja-Bageshwar et al. 2009). **K-Aib-1** also demonstrates potent activity in an in vitro Malpighian tubule secretion assay in mosquito *A. aegypti*, demonstrating a potency that exceeds Aedeskinin-2 and matching that of Aedeskinin-3 (Fig. 2) (Taneja-Bageshwar et al. 2009).

Table 1 Estimated potencies (EC_{50}) and percentage of maximal bioluminescence response of different analogs in reference to FFFSWGa, and the minimum active core sequence FFSWGa, tested on mosquito (E10) and tick (BmLK3) receptor expressing cell lines (Taneja-Bageshwar et al. 2008b, 2009). EC_{50} values are an estimate of the concentration required to induce a half-maximal response

Analogs	Mosquito receptor (E10 cell line)		Tick receptor (BmLK3 cell line)	Selectivity	
	EC_{50} (10–8M)	[a]Maximal Response (%)	EC_{50} (10^{-8}M)	[a]Maximal Response (%)	
Double-Aib Analog					
[Aib]FF**[Aib]**WGa (K-Aib-1)	4.9	100	8	100	Non-selective
Beta Analogs					
1460 Ac-RFF[β_3**Pro**]WGa	36.7	50	29	72	Non-selective
1656 Ac-RFFP[β_2**Trp**]Ga	N.D.	0	68	57	Selective (tick)
1577 Ac-R[β_3**Phe**]FF [β_3**Pro**]WGa	65.3	65	50	64	Non-selective
1578 Ac-RF[β_3**Phe**-β_3**Pro**]WGa	N.D.	7	69	62	Selective (tick)
Controls					
FFFSWGa	68.5	100	27	100	
FFSWGa IK active core	N.D.	29	59	100	
*Aedes*kinin-2 (mosquito)	16.4	100	–	–	

[a]Maximal response is the maximal bioluminescence response of each analog expressed as a percentage of the maximal response of the known agonist FFFSWGa (positive control) at 10 μM. for tick and mosquito receptors. N.D.: The analog was tested but was not sufficiently active to determine an EC50 value. It should be noted that the NOVOstar bioluminescence method used to evaluate the response of the insect kinin analogs on expressed receptors reported in these studies is less sensitive as compared with a less practical confocal fluorescence cytometry method previously employed (Holmes et al. 2003). The bioluminescence plate assay is between 50- to 70-fold less sensitive. This difference should be taken into account when estimating the potency that these analogs would likely demonstrate in in vitro or in vivo physiological bioassays.

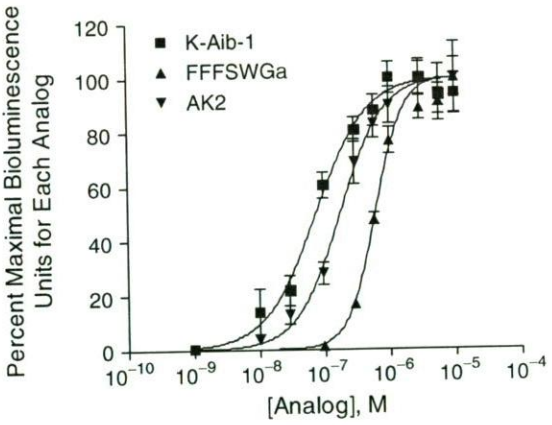

Fig. 1 Activity comparison of biostable analog **[Aib]**FF**[Aib]**WGa (K-Aib-1), native aedeskinin 2, and positive control hexapeptide FFFSWGa on a mosquito kinin receptor expressing cell line by a calcium bioluminescence plate assay (Taneja-Bagehswar et al. 2009c). The y-axis represents percent maximal bioluminescence units for each analog expressed as a percentage of bioluminescence observed at a concentration versus the maximal response observed among all concentrations tested for each analog. Statistical analysis and graphs were created with GraphPad Prism 4.0 software. Vertical lines on graph represent standard errors of independent experiments from maximum of six to minimum of three repetitions each consisting of two wells each. Analog FFFSWGa is the positive control for the receptor activity

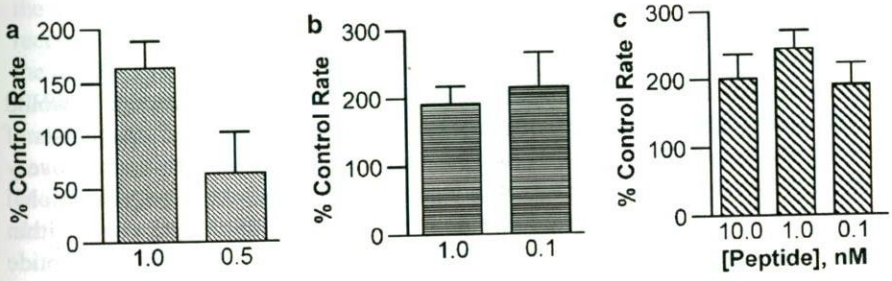

Fig. 2 In vitro *A. aegypti* Malpighian tubule secretion assay of control, FFFSWGa (panel A), native *Aedes* kinin 1 (panel B) and biostable analog **[Aib]**FF**[Aib]**WGa (K-Aib-1) (panel C). Results are expressed as a percentage of the control rate of secretion measured prior to peptide addition. Bars indicate the mean and vertical lines + 1 s.e.m. of 4–5 replicates. (Reprinted from Taneja-Bagehswar et al. (2009) with the permission of Elsevier)

These potent, biostable analogs represent ideal new tools for arthropod endocrinologists studying IK regulated processes, particularly in ticks for which a role for the insect kinins has yet to be established.

While in vivo activity studies of biostable IK analogs have not as yet been completed for the cricket, tick or mosquito, some in vivo results have been obtained in the housefly (*Musca domestica*) and in larvae of the corn earworm moth,

H. zea (Nachman et al. 2002a). Due to the different structure-activity requirements of the IK receptor associated with the Malpighian tubule fluid secretion assay in the housefly (*M. domestica*) (Coast et al. 2002), the in vitro activity of analog 781 is over four orders of magnitude less than that of the native muscakinin. Nonetheless, the in vivo diuretic activity of 781 is *equipotent* with that of the native muscakinin. Evidence indicates that analog 781 demonstrates a longer hemolymph residence time in the housefly than the peptidase-susceptible muscakinin, and it is this extended presence that likely explains the remarkable in vivo activity observed for this analog (Nachman et al. 2002a). Injection of helicokinins into developing larvae of the related moth *H. virescens* has been observed to inhibit weight gain (Seinsche et al. 2000). A helicokinin-II analog (VRFSSWGa) and a biostable Aib helicokinin analog **pQ**RFS**[Aib]**WGa (Hek-Aib) were injected daily (0.5 nmol) into 5-day old *H. zea* larvae for 5 or 6 days until pupation occurred. The helicokinin analog demonstrated a developmental trend that reached a peak on day 5 post-treatment, with a statistically insignificant 20% weight reduction as compared with controls. In contrast, the biostable, helicokinin Aib analog elicited a stronger, statistically significant effect spanning days 4–7, reaching a peak at day 5 of about a 50% reduction in mean larval weight as compared with controls. The time of pupation was delayed by a factor of 25% (Nachman et al. 2002a). Therefore, biostable characteristics can enhance the in vivo activity of IK analogs.

2.3 *Nonpeptide Mimetic Agonists/Antagonists of Expressed IK Receptors*

Perhaps the ultimate goal in the search for biostable, bioavailable analogs would be the design and/or discovery of nonpeptide mimetic agonists or antagonists of the IK. The availability of expressed IK receptors can accelerate the discovery process through the evaluation of nonpeptide libraries. A recent biorational approach has based the selection of a nonpeptide library on the presence, within its structure, of the side chain moiety of the most critical residue of the peptide (R.J. Nachman, uupublished). As discussed in an earlier section, the most critical residues for the interaction of the insect kinins with expressed receptors from the tick *B. microplus* and mosquito *A. aegypti* have been determined to be the Phe_1 and Trp_4 within the C-terminal pentapeptide core region (Nachman et al. 1990; Roberts et al. 1997; Taneja-Bageshwar et al. 2006). Data obtained from an in vitro Malpighian tubule fluid secretion assay indicate that a C-terminal pentapeptide IK analog in which the Phe is replaced with an Ala demonstrates an antagonist response against native achetakinins, whereas the analog in which Trp is replaced with Ala is devoid of activity (Nachman et al. 1993c; Nachman and Holman 1991). Furthermore, a C-terminal aldehyde analog in which Ala replaces Phe retains weak activity in an in vitro cricket diuretic assay (Nachman et al. 2007). This would suggest that Trp, which contains an indole side chain moiety, represents the most critical amino acid for the binding of IK with the receptor.

Consequently, a 400 member nonpeptide library based on the imidopyrido**indole** ('Ipi') scaffold (Reixach et al. 2000), which contains an indole ring embedded in its structure, was constructed and evaluated in expressed insect kinin receptors from the tick *B. microplus* and mosquito *Aedes aegypti*. One of the Ipi recombinant library analogs demonstrated significant activity in both of the expressed arthropod insect kinin receptor assays (R.J. Nachman et al., unpublished), as well as statistically significant activity in in vitro Malpighian tubule secretion assays of the mosquito *A. aegypti* and cricket *A. domesticus* at concentrations between 0.05 and 1 μM (R.J. Nachman et al., unpublished). Future evaluations of this and other rationally designed, indole-containing libraries may identify other nonpeptide mimetic agonists as well as antagonists. The data further underscores the preeminent importance of the indole moiety of Trp_4 to the interaction of the IK with their receptors.

2.4 C-Terminal Aldehyde IK Analogs

Aldehydes can form reversible imine bonds with amino groups. Peptide analogs containing reversible binding moieties, such as an aldehyde, at the C-terminus have been reported to inhibit various classes of proteolytic enzymes (Fehrentz et al. 1984; Chapman 1992; Sarubbi et al. 1993). It has been further postulated that a C-terminal aldehyde moiety could form a covalent, reversible Schiff base (imide linkage) with the amino group of a Lys residue (Lehninger 1970; Nachman et al. 2007) in an IK receptor pocket, thereby modifying the ligand–receptor interaction characteristics of the resulting IK analog. Evaluations of two C–terminal aldehyde IK analogs, R-LK-CHO (Fmoc-RFFPWG-H) and V-LK-CHO (Boc-VFFPWG-H), in developmental and diuretic assays have been reported (Nachman et al. 2003, 2007). Both aldehyde analogs demonstrated in vitro stimulation of fluid secretion in isolated cricket Malpighian tubules in the physiological concentration range and full efficacy, thereby providing evidence that they could interact with an IK receptor site.

2.5 H. Zea Larval Weight-Gain Inhibition Bioassay

Injection of R-LK-CHO into 5-day old *H. zea* larvae induced statistically significant reductions in weight gain in comparison with control animals on days 2 and 4–6 at the 5 nmol dose, but not at 500 pmol. Day 6 larvae experienced a significant reduction in weight gain at the 5 nM dose, with treated animals observed to be about 65% of the weight of controls. No significant difference in mortality was observed between treated and control groups (Nachman et al. 2003). The other aldehyde analog, V-LK-CHO, demonstrated a more pronounced effect than R-LK-CHO in the *H. zea* larval weight gain inhibition assay. V-LK-CHO induced significant reductions in weight gain on days 2, and 4 through 6, after initiation of the treatment at *both* the 500 pmol and

5 nmol dose. At day 6, treated larvae were observed to be 65% and 40% that of the weight of the control animals at doses of 500 pmol and 5 nmol, respectively. Notably, in those animals treated with V-LK-CHO a significant increase in mortality was observed at both doses (45%{500 pmol}and 67%{5 nmol})(Nachman et al. 2003).

The significant increase in mortality observed in larvae treated with V-LK-CHO is not likely a result of some general toxic effect of the aldehyde moiety itself, as increased mortality was not observed in R-LK-CHO, which also features a C-terminal aldehyde (Nachman et al. 2003).

2.6 *In vitro and in vivo Housefly Diuretic Bioassays*

As mentioned previously, IK analogs in which the C-terminal amide was replaced by an aldehyde moiety retained an ability to stimulate fluid secretion by cricket Malpighian tubules. Although a reliable in vivo assay for diuresis in crickets did not exist, such an assay was available for houseflies (Coast 2001, 2004). Neither of the two aldehyde analogs V-LK-CHO and R-LK-CHO stimulated fluid secretion of housefly Malpighian tubules, although notably tubules exposed to R-LK-CHO did not respond when subsequently challenged with a supramaximal concentration (10 nM) of native muscakinin (Musdo-K). In contrast, the same concentration of Musdo-K elicited a marked diuretic response in tubules that had first been exposed to V-LK-CHO. The inhibitory effect of R-LK-CHO on the diuretic activity of 10 nM Musdo-K was dose-dependent with an IC_{50} of 12 μM, which compares favorably with the EC_{50} of an N-terminal truncated Musdo-K analog of similar length (Coast et al. 2002) (Musdo-$K_{[9-15]}$ EC_{50} ~1 μM).

Other aldehyde analogs of the insect kinins were also tested on housefly tubules for diuretic activity and they demonstrated no ability to inhibit stimulation of fluid secretion by 10 nM Musdo-K (Nachman et al. 2007). The inhibitory activity of R-LK-CHO is thus highly specific and not a generalized effect of the aldehyde moiety.

By using a high sensitivity flow through humidity analyzer the excretion of urine from intact houseflies treated with the aldehyde analog and a control was recorded (Coast 2004). R-LK-CHO inhibits the in vivo activity of Musdo-K as evidenced by a marked reduction in the amount of urine voided in flies injected with 50 pmol each of the analog and the kinin compared with the kinin alone (Nachman et al. 2007). The diuretic response to hypervolemia is partly attributable to the release of Musdo-K from neurohaemal sites into the circulation, and the inhibitory effect is consistent with a selective effect of R-LK-CHO at the kinin receptor.

R-LK-CHO would not necessarily be expected to have any effect on the autonomous response of Malpighian tubules to haemolymph dilution, and yet it reduced the total amount of urine voided from flies injected with 1 μL of distilled water by almost 50% (Fig. 3). The markedly reduced urine output from flies injected with 1 μL distilled water containing 50 pmol R-LK-CHO suggests this analog may have a toxic effect on Malpighian tubules. In support of this it has been shown that it not only blocks the activity of Musdo-K, but also blocks

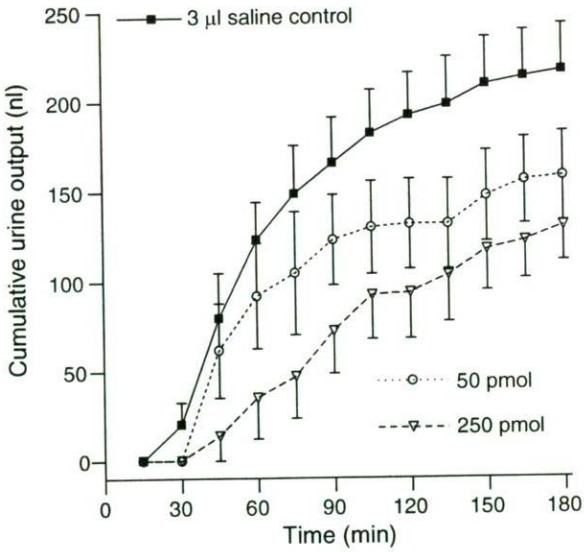

Fig. 3 R-LK-CHO attenuates the in vivo diuretic response to hypervolemia induced by the injection of 3 µL of saline. The volume of urine excreted by individual flies was measured over 15 min periods for 3h in insects injected with saline alone (solid squares, solid line) or saline containing either 50 pmol (open circles, dotted line) or 250 pmol (open triangle, dashed line) of the aldehyde analog. Data points show the means ± 1 S.E.M of the cumulative urine output in 10 (saline alone) and 6 (+ R-LK-CHO) flies. (Reprinted from Nachman et al. (2007) with the permission of Elsevier)

stimulation of fluid secretion by both thapsigargin, a SERCA inhibitor, and by ionomycin, a calcium ionophore (Nachman et al. 2007). Kinin neuropeptides use Ca^{2+} as a second messenger to open a paracellular or transcellular chloride conductance pathway. This is mimicked by thapsigargin and ionomycin, which increase the level of intracellular calcium by promoting Ca^{2+} release for intracellular stores and the influx of Ca^{2+} from the bathing fluid, respectively. The ability of R-LK-CHO to block the activity of these pharmacological probes shows it is not acting as an antagonist of the kinin receptor, but must act downstream of the second messenger pathway.

At present, the cellular action(s) of R-LK-CHO on housefly Malpighian tubules is unknown. It is clearly not a generalized toxic effect, because the same analog has diuretic activity in the cricket Malpighian tubule assay. Moreover, closely related aldehyde analogs tested on housefly tubules, among them those that do not contain the complete IK core sequence, either have no activity or stimulate fluid secretion. R-LK-CHO may act as a 'magic bullet' and bind with the IK receptor on housefly tubules, become internalized, and thereby gain access to intracellular processes that couple a rise in intracellular calcium levels to the opening of the chloride conductance pathway (Nachman et al. 2007).

Compounds in the hemolymph, including pesticide toxins, are actively transported into the lumen of the Malpighian tubules, and their rate of elimination is dependent on

the rate of fluid secretion (Maddrell 1981; O'Donnell and Maddrell 1983). At high rates of secretion, the toxins do not reach the high concentrations in the tubule lumen, which minimizes diffusion back into the hemolymph down a concentration gradient. An agent capable of selective depression of fluid secretion would therefore be expected to reduce the rate of clearance of pesticides from the hemolymph allowing them to achieve higher concentrations and, in turn, likely reduce the amount of toxin required to kill an insect.

3 Pyrokinin/PBAN Neuropeptide Family

The pyrokinin/pheromone biosynthesis activating neuropeptide (PK/PBAN) peptides represent a multifunctional family that plays a significant role in the physiology of insects. Leucopyrokinin (LPK), isolated from the cockroach *Leucophaea maderae* in 1986 (Holman et al. 1986), was the first member of the family to be discovered. Since that time, over 30 peptides have been identified. They include PKs, myotropins (MTs), PBAN, melanization and reddish coloration hormone (MRCH), diapause hormone (DH), pheromonotropin (PT), all of which share the common C-terminal pentapeptide FXPRL-amide (X = S, T, G or V) (Altstein 2004; Predel and Nachman 2006; Rafaeli and Jurenka 2003). Functions of the PK/PBAN family include stimulation of sex pheromone biosynthesis in moths (Altstein 2004; Matsumoto et al. 1992; Predel and Nachman 2006; Raina and Klun 1984; Rafaeli and Jurenka 2003; Raina et al. 1989), and mediation of key aspects of feeding (gut muscle contractions) (Nachman et al. 1986; Schoofs et al. 1991), development (embryonic diapause, pupal diapause and pupariation) (Imai et al. 1991; Nachman et al. 1993a, 1996, 1997b, 2006; Xu and Denlinger 2003; Zdarek et al. 2002) and defense (melanin biosynthesis) (Altstein et al. 1996; Matsumoto 1990) in a variety of insects (cockroaches, flies, locusts and moths). All of the above functions can be stimulated by more than one peptide, and they demonstrate considerable cross-activity between various PK/PBAN assays, thereby lacking any species-specific behavior (Abernathy et al. 1996; Altstein 2003; Choi et al. 2003; Nachman et al. 1993a; Rafaeli and Jurenka 2003). The functional diversity of the PK/PBAN family raises many questions regarding the mechanisms by which these neuropeptides elicit their effects. Agonists and/or antagonists, particularly selective ones, of the PK/PBAN family can shed light on this issue, and provide leads for insect management agents capable of disrupting PK/PBAN regulated systems.

3.1 Chemical and Conformational Aspects of PK/PBAN Activity

The C-terminal pentapeptide FXPRLa is highly conserved and thus, shared by PBAN and other pyrokinins. It has further been identified as the ligand core for activity in pheromonotropic bioassays (X = S) (Abernathy et al. 1995; Altstein et al. 1995) and the expressed PBAN receptor from the moth *H. virescens*

(HevPBANR-C) (Kim et al. 2008), although the C-terminal hexapeptide YFXPRLa (X = S) exhibits much greater potency. The C-terminal pentapeptide also has been shown to represent the core region for activity retention in such other PK/PBAN mediated physiological systems as the cockroach *L. maderae* hindgut contractile (Nachman et al. 1986) and melanization assays in *Spodoptera littoralis* (Altstein et al. 1996). However, the core region for activity is the C-terminal heptapeptide LWFGPRLa in the pupal diapause termination bioassay in *H. zea* (Zhang et al. 2008) and the C-terminal tetrapeptide TPRLa in the pupariation bioassay in the fly *Neobellieria bullata* (Nachman et al. 1997).

Several turn conformations have been proposed for the pentapeptide region of the PK/PBAN core based on NMR experiments of PBAN and/or core analogs in solution. Using the C-terminal hexapeptide PBAN analog [D-Phe$_{29}$, 28–33]PBAN in an NMR solution conformation study, Wang et al. reported that it adopts a type II β-turn; although the authors concluded that this may have resulted from the conformational averaging of a type I β-turn and an extended structure (1994). Clark and Prestwich investigated the solution conformation of the natural HezPBAN and reported a type I' β-turn with a *cis*-Pro in the C-terminal pentapeptide region (1996). They found no interaction between the C-terminal turn and the rest of the PBAN peptide chain, providing evidence that the turn is the critical conformation recognized by the PBAN receptor. Drawbacks to the studies conducted by Wang et al. and Clark and Prestwich are that they were investigating highly flexible structures and NMR experiments were conducted in solutions containing organic solvents (Wang: DMSO; Clark/Prestwich: 2,2,2-trifluoroethanol), which can promote formation of secondary structure that is not necessarily relevant to the conformation adopted during receptor docking. Nachman et al. conducted a conformational study of the rigid, cyclic PK/PBAN analog *cyclo*[NTSFTPRL] (*cyclo*[Asn1]LPK) in aqueous solution containing no organic solvents using a combination of NMR spectroscopic and molecular dynamics (Nachman et al. 1991, 2003b). The specific conformation of this constrained, cyclic analog in aqueous solution was shown to be extremely rigid, featuring a *trans*-oriented Pro in the second position of a type-I β-turn over residues Thr-Pro-Arg-Leu within the core region. Indeed, a *trans*Pro is a defining characteristic of a type I β-turn (Chou and Fasman 1977). The very large (for Thr-2, Thr-5, and Leu-8) and very small (for Ser-3 and Arg-7) coupling constants found indicated that the backbone of *cyclo*[Asn1]LPK was rigidly held in a single or a few closely related conformations, since conformational averaging would have given averaged, intermediate values (Nachman et al. 1991). This analog (*cyclo*[Asn1]LPK) demonstrated significant activity in several PK/PBAN bioassays, including hindgut contractile (cockroach *L. maderae*) (Nachman et al. 1991), oviduct contractile (cockroach *L. maderae*) (Nachman et al. 1995), pheromonotropic (silk worm *Bombyx mori*) (Nachman et al. 2003b), egg diapause induction (silk worm *B. mori*) (Nachman et al. 1995), pupal diapause termination (*H. zea*) (Zhang et al. 2009), and pupariation (flesh fly *N. bullata*) (Nachman et al. 1997) assay systems. Recently, a structure for the HezPBAN receptor has been predicted using the X-ray diffraction structure of the GPCR rhodopsin as a template; and this calculated structure has been used to build a binding model for the HezPBAN C-terminal hexapeptide fragment adopting each of the three

proposed β-turn types. The model clearly supports the presence of a β-turn in the receptor bound conformation of the PBAN core, but is not precise enough to provide evidence for the specific type of β-turn (Stern et al. 2007).

In order to provide more definitive evidence that a *trans*Pro, and a type I β-turn, represented the active conformation for pheromonotropic activity, a PK/PBAN analog incorporating a *trans*Pro, (*E*)-alkene mimetic component was evaluated in five PK/PBAN bioassay systems (pheromonotropic, pupariation, hindgut myotropic, and melanization) and found to retain high activity (Nachman et al. 1995, 2009f). The (*E*)-alkene mimetic analog demonstrated activity that was not statistically different from that of a PK/PBAN C-terminal pentapeptide parent analog in an expressed HevPBANR receptor (Nachman et al. 2009c) and proved to be an order of magnitude more potent than the native 24-residue diapause hormone (DH) in the pupal diapause termination assay in *H. zea* (Zhang et al. 2009). The greater potency observed in the latter in vivo assay is believed to be due to greater biostability of the mimetic analog to degradative peptidases. In the (*E*)-alkene moiety of the aforementioned PK/PBAN analog, the peptide bond that binds the amino group of the Pro is locked into a *trans* orientation by replacement with a double bond, which lacks the ability to rotate between *trans* and *cis* orientations as does a normal peptide bond (Wang et al. 2003).

The establishment of a *trans*Pro orientation has important implications concerning the preferred β-turn conformation. Previous NMR studies have led to the three proposed β-turn types (type I, type II and type I′) for the PBAN core region discussed above. Of the three studies, only Nachman et al. (1991, 2003b) used both a conformationally rigid PK/PBAN analog along with aqueous solutions free of added organic solvents that artificially promote the formation of secondary structure. Of further interest is the admission by Wang et al. (1994) that their finding of a type II β-turn could have resulted from the conformational averaging of a type I β-turn (identified in the study by Nachman et al.) and an extended conformation in the flexible analog used. The type I β-turn proposed by Nachman et al. features a *trans*Pro that was clearly evident in the rigid, cyclic analog *cyclo*[Asn1]LPK and has now been confirmed by the potent activity of the (*E*)-alkene analog, which locks in a *trans* orientation. This finding is not consistent with the type I' β-turn proposed in the study by Clark and Prestwich (1996) that used the highly flexible HezPBAN as it features a *cis*Pro as opposed to a *trans*Pro. The activity of the (*E*)-alkene analog not only provides evidence for the orientation of Pro and a core conformation for the interaction of PK/PBAN peptides with their receptors, but also identifies a scaffold with which to design mimetic PK/PBAN analogs.

3.2 *Development of a Selective PK/PBAN Agonist Analog*

While it had been established that Pro in the ligand core of PK/PBAN analogs preferentially adopts a *trans* orientation during receptor interaction, it was unknown whether differences existed between the PK/PBAN receptors associated with the

various bioassay systems in terms of tolerance to any variance from the natural *trans*Pro conformation. Any differences could be exploited to develop mimetic analogs with selectivity for one of the related PK/PBAN receptors over others. In this regard, the dihydroimidazoline moiety (Fig. 5) was proposed as a novel mimic of a *trans* peptide bond, particularly one associated with Pro, and then incorporated into a PK/PBAN core sequence. Molecular modeling indicated that the dihydroimidazoline moiety (labeled 'Jones' moiety; abbreviated 'Jo') could serve as a surrogate of a *trans* peptide bond and/or a *trans*Pro, although the fit is not as close as with the (*E*)-alkene moiety above (Nachman et al. 2009a). An analog incorporating the 'Jones' moiety **PPK-Jo**, Ac-YF[**Jo**]RLa, was evaluated in four PK/PBAN assays (pheromonotropic, melanotropic, pupariation and hindgut myotropic). In the in vivo *S. littoralis* melanotropic assay **PPK-Jo** demonstrated strong activity, reaching a 100% maximal response at doses of 100 pmol and 1 nmol; matching the efficacy of the natural peptide HezPBAN1–33 if not its potency. The parent PK/PBAN hexapeptide YFTPRLa demonstrated a strong response in this assay as well,

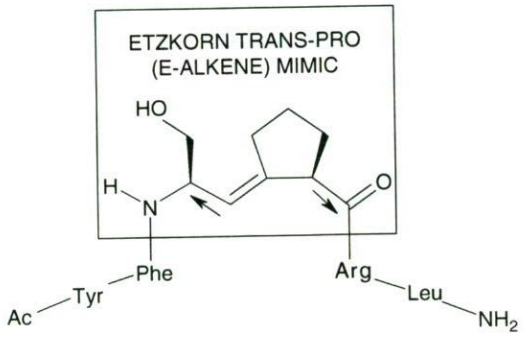

Fig. 4 Structure of the analog PK-Etz (Ac-Tyr-Phe-SerΨ[***trans-CH = C***]Pro-Arg-Leu-NH2), containing an (E)-alkene, *trans*Pro motif ('Etzkorn'). In this motif, the peptide bond that binds the amino group of the Pro is locked into a *trans* orientation by replacement with a double bond, which lacks the ability to rotate between *trans* and *cis* orientations as does a normal peptide bond. (Wang et al. 2003; Nachman et al. 2009a)

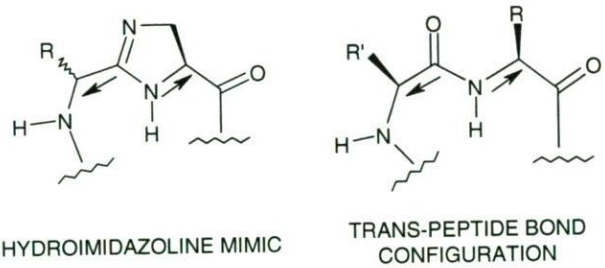

Fig. 5 Comparison of the *trans*-peptide bond (right) and the dihydroimidazoline ('Jones') mimetic motif. A '*trans*' orientation is frozen within the five-membered ring structure (Nachman et al. 2009a)

but, unlike **PPK-Jo** it also inhibited the melanotropic activity of control peptide HezPBAN1–33. Therefore, **PPK-Jo** proved to be a pure agonist in the melanotropic assay. **PPK-Jo** did not elicit any significant agonist or antagonist activity in the pheromonotropic, pupariation or hindgut contractile bioassays, indicating that it is a pure, selective agonist for the melanotropic response in *S. littoralis* (Nachman et al. 2009a). In contrast, control peptides elicited strong agonist responses in all three of these PK/PBAN bioassays. It is apparent that the receptor associated with the melanotropic assay in *S. littoralis* is more promiscuous than those of the other PK/PBAN assays, demonstrating more tolerance to deviations from the natural *trans*Pro structure. The development of a selective PK/PBAN agonist can lead to a better understanding of the endogenous mechanisms of this important peptide class and can serve as a probe to study PK/PBAN regulated systems in insects and as a lead for insect management agents capable of disrupting these systems in a selective manner.

Another analog incorporating a structural modification at the Pro residue of the PK/PBAN core region that features a selective agonist profile is covered under the next section on biostable analogs of the PK/PBAN family. In other work, Altstein et al. investigated cyclized versions of the linear lead PK/PBAN antagonist RYF[dF]PRLa that feature selective inhibition of either the pheromonotropic or melanotropic activity of control PK/PBAN peptides (Altstein 2003; Altstein and Hariton 2008). This work is covered extensively in another chapter of this book (Altstein et al. this book), and therefore will not be further addressed here.

3.3 *Biostable PK/PBAN Analogs*

The PKs are hydrolyzed by tissue-bound peptidases at a primary susceptibility site between the Pro and Arg residues within the C-terminal pentapeptide sequence that defines members of this family of neuropeptides (Nachman et al. 2002b). Incorporation of β-amino acids can enhance resistance to peptidase attack and modify biological activity (Cheng et al. 2001; Juaristi and Soloshonok 2005), as demonstrated in the first section of this chapter with the insect kinin neuropeptide family (Taneja-Bageshwar et al. 2008b; Nachman et al. 2009d). This strategy can lead to the identification of potent selective and nonselective agonists with enhanced biostability characteristics. Analogs of the PK/PBAN C-terminal hexapeptide core (YFTPRLa) that incorporated β_3-Pro as a replacement for Pro were synthesized with the expectation that this modification could protect the adjacent peptidase-susceptible peptide bond linking the Pro and Arg residues. The two analogs, **PK-βA-1** (**Ac**-YFT[β_3**P**]RLa) and **PK-βA-4** (**Ac**-[β_3**F**]FT[β_3**P**]RLa), were tested against two peptidases (neprilysin and angiotensin-converting enzyme [ACE]) that degrade and inactivate natural PK/PBAN peptides and found to demonstrate greatly enhanced resistance to hydrolytic cleavage. For neprilysin, the observed rates of hydrolysis for the parent peptide YFTPRLa, **PK-βA-1**, and **PK-βA-4** were found to be about 700, 0 and 30 pmol/h, respectively. For ACE, the rates of hydrolysis were observed to be

about 2,360; 0 and 210 pmol/h, respectively (Nachman et al. 2009b). **PK-βA-1** is a particularly biostable analog to these two degradative peptidases. Despite the insertion of an additional methylene group into each of two residues of the PK/PBAN core region, analog **PK-βA-4** demonstrates an agonist response in all four of the PK/PBAN bioassays in which it was evaluated (pheromonotropic, melanotropic, pupariation, and hindgut myotropic), but is especially potent in the *N. bullata* pupariation assay, where it essentially matches the potency of a natural control PK. Analog **PK-βA-1** demonstrates a more selective profile than control PK/PBAN peptides as it is essentially inactive in the pupariation bioassay and represents a biostable antagonist of the pheromonotropic and melanotropic assays, without eliciting the significant agonist activity of the parent PK/PBAN hexapeptide.

Other β-analogs of the PK/PBAN family with either a β_3-Phe or β_2-homo-Phe replacement for the Phe, a residue far removed from the primary peptidase-susceptible site, were not expected to feature the extent of biostability observed for the aforementioned β_3-Pro analogs but did modify the biological activity profile of the parent PK/PBAN hexapeptide. Analog **PK-βA-2** (**Ac**-Y[**$\beta_2$homoF**]TPRLa) proved to be an especially potent agonist in the in vivo *S. littoralis* melanotropic bioassay, demonstrating full efficacy at 1 pmol. Analog **PK-βA-3** (**Ac**-Y[**β_3F**]TPRLa) was found to be a non-selective agonist in all four PK/PBAN bioassays, but elicits a particularly unusual response in the *N. bullata* pupariation bioassay (Nachman et al. 2009b). **PK-βA-3** accelerates pupariation in this fly at a potency (0.2 pmol/larva) that matches that of the native pyrokinin factor. At higher concentrations, this β-amino acid pyrokinin analog induces irregular pupariation behavior patterns that are suggestive of neurotoxic properties. Specifically, the immobilization (*I*) phase began almost immediately after recovery from chilling (there was no wandering) and violent convulsive contractions of overall musculature were observed during the *I* phase (Nachman et al. 2009f). Additional modifications intended to protect the primary peptidase-susceptible peptide bond that links the Pro and Arg residues of **PK-βA-3** may lead to biostable analog leads with the potential to further disrupt the important pupariation process in flies.

Other PK/PBAN analogs have been synthesized that feature sterically-hindered replacements for Pro in the core region to enhance resistance to peptidase hydrolysis at the adjacent primary peptidase-susceptible site. The Pro analogs hydroxyproline (Hyp) and octahydroindole-2-carboxylate (Oic) were incorporated into the core region of the PK/PBAN pentapeptide core region. The resulting analogs, **Hex**-FT**[Hyp]**RLa (901) and **Hex**-FT**[Oic]**RLa (904), proved to be completely resistant to degradation by peptidases bound to Heliothine Malpighian tubule tissue over a 120 min period, whereas a natural pyrokinin was completely degraded in 30 min. Despite the structural changes incorporated into the biostable Hyp (901) and Oic (904) analogs, they retained significant activity in a *H. virescens* pheromonotropic assay, with ED_{50} values of 9 and 114 pmol, respectively (Nachman et al. 2002b). The ED_{50} of the parent PK/PBAN pentapeptide FTPRLa was 2.3 pmol. The two analogs matched the efficacy of natural HezPBAN and/or its fragment-analogs. Although Oic analog 904 was inactive in a *H. zea* pupal diapause termination assay, Hyp analog 901 proved to be 5-fold more potent than the native DH hormone (Zhang et al. 2009). The enhanced

potency of the biostable analog is likely a result of the longer in vivo half life as compared with the native hormone. Therefore, the Hyp analog 901 is a biostable, *nonselective* agonist for both the pheromonotropic and pupal diapuase termination response in Heliothine insects, whereas Oic-containing 904 is a biostable analog that is *selective* for the pheromonotropic bioassay.

3.4 PK/PBAN Analogs with Enhanced Topical and/or Oral Bioavailability

Insect neuropeptides in general are not suitably designed to efficiently penetrate either the outer cuticle or the digestive tract of insects. Nonetheless, studies have shown that the PK/PBAN class of insect neuropeptides can be modified to enhance bioavailability characteristics.

3.5 Topical Activity

Topical experiments have been conducted with a couple of insect neuropeptide families. Topical application of members of the adipokinetic hormone (AKH) family in mixed aqueous/organic solvent to the cuticle of the cricket *Gryllus bimaculatus* did lead to a significant AKH-like increase in hemolymph lipids (Lorenz et al. 2004). The presence of organic solvent likely served as a carrier for the AKH peptides in penetrating the cuticular waxes. In addition, the AKHs are particularly hydrophobic, which may also aid penetration through the cuticle.

On the other hand, experiments involving topical application of aqueous solutions of members of the PK/PBAN family did not produce significant pheromone production in the tobacco budworm moth *H. virescens* (Nachman et al. 1996; Abernathy et al. 1996). Structural modification to produce PK/PBAN analogs that feature amphiphilic properties greatly enhances their ability to both penetrate the hydrophobic cuticle, and also to maintain the aqueous solubility required to reach their target receptor once they encounter the hemolymph (Nachman et al. 1996; Abernathy et al. 1996). The development of a series of pseudopeptide analogs of this neuropeptide family was accomplished with the addition of various hydrophobic groups to the N-terminus of the C-terminal pentapeptide active core, which in conjunction with the polar/charged Arg side chain, confer an amphiphilic property. Hydrophobic groups appended to the N-terminus included fatty acids of various chain lengths, cholic acid, carboranylpropionic acid, and aromatic acids (Nachman et al. 1996, 2001, 2002b; Nachman and Teal 1998; Teal and Nachman 1997,2002). Many of these amphiphilic analogs showed greater in vivo potency in a pheromonotropic assay than the native 33-membered PBAN when delivered via injection in female *H. virescens* moths. In studies involving topical application in aqueous solution, neither PBAN nor its C-terminal pentapeptide active core

elicited pheromone production when applied at 1–2 nmol/female. By contrast, various amphiphilic analogs induced significant pheromone production 15 min after topical application of aqueous solutions to the lateral abdominal surface of the moths with ED_{50} values ranging from 60 to 500 pmol/female and ED_{max} values of 60–2,000 pmol/female (Nachman and Teal 1998), dependant on the individual analog. No assistance by organic solvents was needed. Following application to dissected pieces of *H. virescens* cuticle, 24-h recoveries of a series of amphiphilic pyrokinin analogs ranged from 5–70%, depending on the individual analog. In addition, prolonged pheromone production exceeding 20 h was observed following a single topical application of one amphiphilic PK/PBAN analog to the moth *H. virescens*. Furthermore, the nature of the hydrophobic moiety was observed to influence the duration of the slow release of a particular amphiphilic analog. It is clear that the insect cuticle can serve as a reservoir for the time-release of a physiologically active, amphiphilic analog of an insect neuropeptide (Nachman and Teal 1998).

One amphiphilic PK/PBAN analog, **2Abf-Suc**-FTPRLa (PK-2Abf), featured an appended brominated fluorine aromatic ring as the hydrophobic moiety and demonstrated highly unusual in vivo activity following delivery via injection (Teal and Nachman 2002). Unlike other amphiphilic analogs, a single injection of 500 pmol of this brominated fluorine (2Abf) pyrokinin analog into female *H. virescens* moths induced a highly unnatural response; continuous production of high levels of pheromone for as long as 20 h (Teal and Nachman 2002). While such a result might be expected from the time-release of an amphiphilic analog following topical application, the observed prolonged pheromone production following injection suggested that the 2Abf analog might have a strong affinity for the pheromone receptor. Studies on an expressed PK/PBAN receptor from *H. virescens*, the Abf analog proved to be more active than the native 33-membered PBAN neuropeptide, and much more active than the parent C-terminal pentapeptide fragment (R.J. Nachman and M.E. Adams, unpublished). When evaluated in an in vivo pupal diapause termination assay in *H. zea*, this Abf analog proved to be hyperpotent, with an EC^{50} nearly 50-fold more potent than the natural DH hormone (Zhang et al. 2009). However, in the pheromonotropic assay the analog had an interesting side effect in that it led to mortality in 100% of the treated *H. virescens* adults. The LC^{50} value for this potent toxic side effect was found to be 0.7 pmol, whereas 100% mortality was achieved with a 5 pmol dose. Related analogs such as **2Abf-Suc**-AARAAa, retaining similar amphiphilic and solubility properties, failed to demonstrate toxicity (Teal and Nachman 2002). Therefore, the effect was not a result of any inherent toxicity of the 2Abf moiety itself. Furthermore, the toxic effect was highly specific to the presence of the PK/PBAN sequence. Although the mechanism of the insecticidal activity of the PK-2Abf analog in *H. virescens* is unknown, it is hypothesized that the toxicity results from an interaction with receptor sites for the PK/PBAN class of insect neuropeptides (Teal and Nachman 2002).

The same strategy used to develop PK/PBAN agonists with enhanced bioavailability characteristics was recently employed with the linear lead PK/PBAN *antagonist* RYF[dF]PRLa (Ben-Aziz et al. 2005; Zeltser et al. 2000). Addition of the hydrophobic hexanoic acid to the N-terminus led to the amphiphilic analog

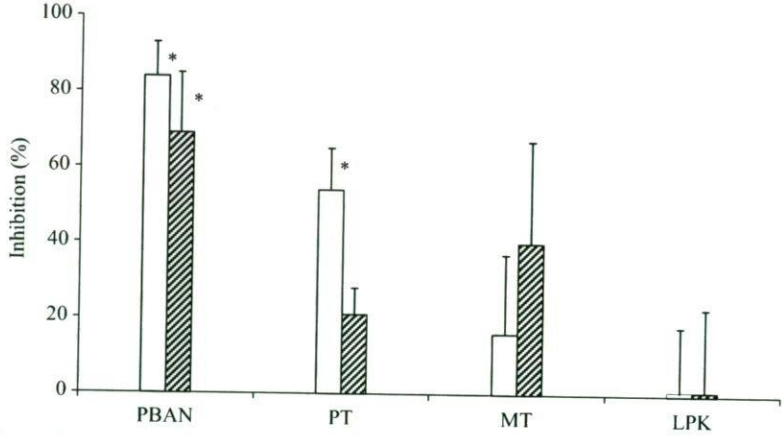

Fig. 6 In vivo inhibition of sex pheromone biosynthesis elicited by PBAN, PT, MT and LPK by 100 pmol (*open bars*) and 1 nmol (*dark bars*) of the amphiphilic analog PPK-AA in adult female *H. peltigera*. The data represent means ± SEM (n = 8–10). An asterisk (*) indicates an activity that differs significantly (at $P < 0.05$) from that obtained by the elicitor itself. (Reprinted from Nachman et al. (2009e) with the permission of Elsevier)

Hex-Suc-A[dF]PRLa (PPK-AA), which was able to inhibit the pheromonotropic activity of PBAN by a factor of 84% at a dose of 100 pmol in *H. peltigera* (Fig. 6). PPK-AA is a pure, selective antagonist as it demonstrates no significant agonist response in either the heliothine pheromonotropic assay or in the *S. littoralis* melanotropic assay and no inhibition of control PK/PBAN peptides in the melanotropic assay. Testing on isolated cuticle dissected from the abdominal region of adult heliothine females demonstrates that from an initial topical application of 500 pmol, a high percentage (25–30%, or 130–150 pmol) penetrates through the outer cuticular surface to the hemolymph side (Fig. 7) (Nachman et al. 2009e). The cuticle-penetration data indicates that the quantity of the amphiphilic antagonist PPK-AA capable of transmigration through the abdominal heliothine cuticle is sufficient to reach a physiologically significant dose. The amphiphilic analog PPK-AA is a selective pheromonotropic antagonist featuring enhanced bioavailability, and represents a significant addition to the arsenal of tools available to arthropod endocrinologists studying the endogenous mechanisms of PK/PBAN regulated processes, as well as a prototype for the development of environmentally friendly pest management agents capable of disrupting the critical process of reproduction.

3.6 Oral Activity

Generally, oral activity for unmodified insect neuropeptides is poor to nonexistent. Small quantities of members of the PK/PBAN (Raina et al. 1995) and the proctolin classes of neuropeptides (Bavoso et al. 1995) have been previously reported to

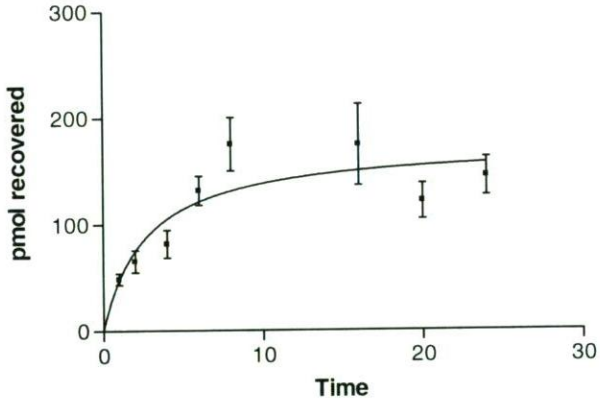

Fig. 7 Penetration of the amphiphilic antagonist analog PPK-AA through the isolated cuticle dissected from the abdominal region of adult female *H. virescens* at 1, 4, 6, 8, 16, 20, and 24 h intervals, as monitored via HPLC. The data represent means ± SEM (n = 6). (Reprinted from Nachman et al. (2009e) with the permission of Elsevier)

survive exposure to the digestive enzymes of the digestive tract and penetrate through to the hemolymph to reach their target receptors. In addition, small quantities (<3%) of A-type allatostatins have been shown to be transported across dissected foregut tissue of the moth *Manduca sexta* (Audsley and Weaver 2007). An attempt to feed PBAN to adult females of the moth *H. zea* led to very low and inconsistent levels of pheromone production that were not progressively dose-dependent (Raina et al. 1995). In other experiments, no statistically significant pheromone production was observed in starved adult females of the related moth species *H. virescens* 1–2 h after ingestion of a sugar solution of 50 pmol/μL of PBAN or the C-terminal pentapeptide core FTPRLa. However, the aforementioned biostable amphiphilic, pyrokinin analogs **Hex**-FT**[Hyp]**RLa (901) and **Hex**-FT**[Oic]**RLa (904) demonstrated an ability to penetrate the dissected portions of the insect digestive tract as well as elicit significant pheromonotropic activity following oral delivery (Nachman et al. 2002c).

Direct penetration of the two analogs 901 and 904 through dissected cockroach foregut and midgut were investigated. The digestive system of the cockroach was chosen because the guts of adult moths are not of sufficient size or stability to allow for practical delivery of peptide analog solutions. Out of a total of 2.5 nmol placed within the lumen of a sealed foregut, 800 nmol (over 30%) of Oic analog 904 penetrated the tissue preparation. It is interesting to note that Oic analog 904 demonstrates time-release properties, as equal amounts were recovered over the 0–4 h period as over the 4–24 h period. The majority of Hyp analog 901 penetrated in the first 0–4 h period (Nachman et al. 2002c). The lumen of the insect foregut features a cuticular component, which could explain why the time-release effect is similar to that observed for the outer cuticle for these amphiphilic analogs. It also suggests that the foregut can serve as a reservoir for the time-release delivery of neuropeptide analogs in insects, thereby bypassing the hostile, peptidase-rich environment of the midgut.

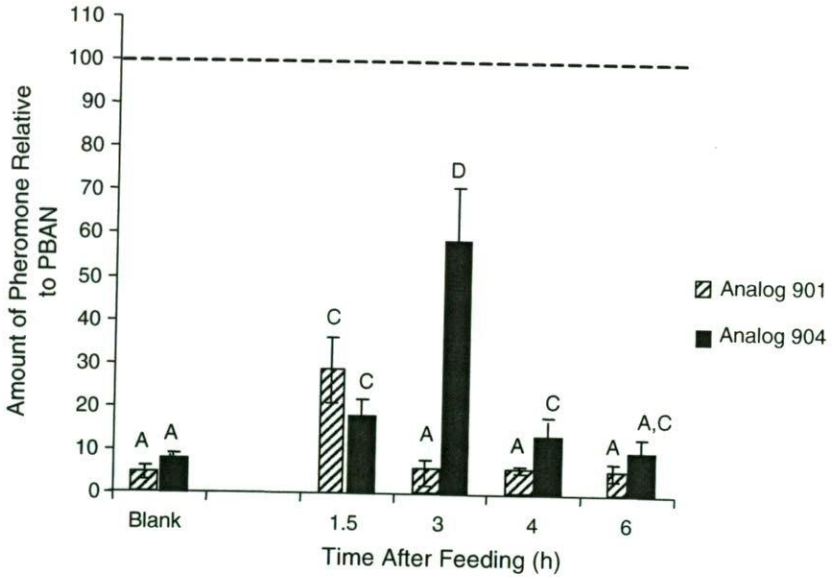

Fig. 8 Amount of pheromone, relative to maximal levels produced by injected PBAN, produced by Hyp-pyrokinin analog 901 and Oic-pyrokinin analog 904, at 1.5, 3, 4, and 6 h following oral administration. The dotted line at 100% denotes maximal production of pheromone by injected PBAN (positive control). (Reprinted from Nachman et al. (2002c) with the permission of Elsevier Press)

These in vitro penetration studies of analogs 901 and 904 were followed by in vivo oral pheromonotropic activity trials in adult female *H. virescens* (Nachman et al. 2002c). Pheromone production was monitored following ingestion of 30 μL of a sugar solution containing 50 pmol/ μL of either 901 or 904 at 1.5, 3, 4 and 6 h post feeding (Nachman et al. 2002b). A statistically significant increase in pheromone titer was observed at 1.5 h post-feed with 901 with a 17% maximal response. Oral administration of the analog 904 induced statistically significant levels of pheromone at 1.5, 3 and 4 h post-feed, but not at 6 h. Optimal pheromone production was achieved at 3 h, with a highly significant 60% maximal response (Nachman et al. 2002c). The shift in the pheromone spike from 1.5 h for 901 to 3 h post-feed for 904 is consistent with the greater time-release effect observed for the direct penetration of the more hydrophobic 904 in both ligated fore-and midgut preparations.

4 Summary

Structure-activity studies employing restricted-conformation analogs have led to a greater understanding of the chemical and conformational aspects of the interaction of the IK and PK/PBAN neuropeptide classes with expressed arthropod receptors.

In the process, several turn-mimic motifs have been identified as scaffolds for the development of mimetic agonist and antagonist neuropeptide analogs with enhanced biostability. These include the tetrazole and Apy *cis*Pro-mimetic motifs for the IK family and (*E*)-alkene and dihydroimidazoline *trans*Pro-mimetic motifs for the PK/PBAN class. Biostable mimetic analogs of both neuropeptide families have been shown to match or exceed the in vitro and in vivo activity, disrupt normal IK or PK/PBAN neuropeptide-regulated physiological processes, feature enhanced selectivity for particular physiological processes and/or species specificity, and in some cases result in increased mortality in insects. A 'magic bullet' C-terminal aldehyde IK analog selectively targets housefly Malpighian tubules, the major organ of diuresis in insects, and leads to marked inhibition of urine release. An agent capable of selective depression of fluid secretion would be expected to allow pesticides to achieve higher concentrations in the hemolymph; and, in turn, likely reduce the amount of toxin required to kill an insect.

While neuropeptides are not generally designed for penetration of the outside cuticle or the gut wall in large quantities, enhancement of bioavailability has been demonstrated in the PK/PBAN neuropeptide class. Amphiphilic agonist PK/PBAN analogs have shown an ability to efficiently penetrate in vitro preparations of insect cuticle and foregut, as well as demonstrate potent activity in in vivo pheromonotopic bioassays when administered via topical or oral routes. An amphiphilic PK/PBAN analog that features a pure, selective antagonist profile in a heliothine pheromonotropic bioassay demonstrates an ability to penetrate in vitro preparations of heliothine cuticle in quantities that have been shown to inhibit the pheromonotropic activity of PBAN by 84%.

In conclusion, the studies presented here have led to the identification of interesting tools for arthropod endocrinologists and promising mimetic analog leads that when either delivered in isolation, or possibly in concert with mimetic analogs of other neuropeptide classes that regulate different critical physiological processes, may be effective in managing arthropod pests.

Acknowledgments The financial assistance from the USDA/DOD DWFP Initiative (#0500–32000–001–01R), a US-Israel Binational Agricultural Research and Development Fund (BARD) grant (IS-3356–02), a North Atlantic Treaty Organization (NATO) Collaborative Research Grant (#LST.CLG.979226), and a Texas Advanced Technology/Research Grant (#000517–0103–2001) is acknowledged.

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